Office of Naval Research Annual Report
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a. Scientific and Technical Objectives (<200 words)

Our overall objective is to facilitate the rational design of biosynthetic pathways for the production of unnatural compounds. We are developing tools and attempting to elucidate rules to establish a framework for "retro-biosynthesis," the practice of rationally proposing a synthetic scheme for a target compound from one or more starting substrates, based only on enzyme-mediated transformations. We are especially focused on experimental realization of microbial synthesis, and the limits thereof, in contrast to theoretical predictions. A critical tool is protein engineering for altered substrate specificity, either through rational protein design or random mutagenesis (directed evolution).

We have chosen glucaric acid as a model compound for pathway proposition and assembly in *Escherichia coli*. This compound is naturally-occurring in plants and mammals, but a microbial pathway has not been established. Specific objectives are to: (1) propose several pathways for glucaric acid synthesis, (2) select at least one pathway for experimental study, and (3) establish microbial synthesis of glucaric acid. A fourth objective arising from this work is the development of a database for the re-classification of enzyme activities based only on substrate and product functional groups. The database is designed to enable searches more amenable to enzyme selection for biosynthetic pathway proposition.

b. Approach (<200 words)

We propose pathways using an approach that is analogous to that used by organic chemists in proposing synthesis schemes. We have proposed pathways towards glucaric acid that utilize only enzymes by searching the available databases for both specific conversions (e.g., the production of glucaric acid from glucuronic acid by *Pseudomonas syringae* uronate dehydrogenase) and generalized enzyme functions (e.g., the conversion of an aldehyde to a carboxylic acid through EC class 1.2.1 enzymes). We do not attempt an exhaustive search of all theoretical conversions, but rather focus on those most likely realizable based on the prevalence of enzymes within a certain reaction class. Given a pathway design, we attempt construction by recruiting enzyme activities through PCR amplification of known genes, chemical synthesis of codon-optimized DNA fragments for expression in *E. coli*, and cloning of un-sequenced genes using genomic DNA libraries and/or protein purification. Successful construction of some pathways will require enzyme engineering to achieve desired conversion steps.

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We have identified one potential pathway that utilizes only naturally-occurring enzymes but from disparate sources. A second pathway has been designed and requires enzyme engineering for two of three reaction steps. We have established collaborators in protein design to achieve this goal.

c. Concise Accomplishments (200 words)

- **Cloning uronate dehydrogenase activity from *Pseudomonas syringae*** – We have utilized a previously developed screening method to isolate the gene encoding uronate dehydrogenase (*udh*) from a genomic DNA library. This enabled the successful demonstration of the benchmark pathway for glucaric acid production.

- **Assembly of the benchmark pathway** – Three enzymes (MIPS from yeast, MIOX from mouse, and UDH from *P. syringae*) were co-expressed in *E. coli* to successfully produce glucaric acid. Modulation of inducer concentrations resulted in titers of ~1 g/L. This is the first demonstration of glucaric acid production in a microbial host.

- **Improvement of productivity through co-localization of enzymes** – Through a collaboration with Dr. John Dueber at the University of California, Berkeley, we utilized synthetic scaffolds to co-localize the MIPS and MIOX proteins. This resulted in an improvement of glucaric acid titer up to ~2 g/L.

- **Collaboration for protein design** – We received the first library to screen for a new glucose oxidase activity from our industrial collaborator. This will result in the identification of improved enzyme activities for use in the designed pathway.

- **Modeling designed pathways** – Through an academic collaboration, we have developed a simple model to estimate the metabolic burden associated with proposed novel metabolic pathways.

d. Expanded Accomplishments

- **Cloning uronate dehydrogenase activity from *Pseudomonas syringae*** – At the end of the last reporting period, we had unsuccessfully screened several open reading frames from *P. syringae* with putative 1.2.1 activity, capable of converting an aldehyde to a carboxylic acid. We had also obtained a synthesized version of a gene sequence deposited in GenBank and annotated as a "uronate dehydrogenase." At the beginning of the current reporting period, testing of this recombinant enzyme under a variety of conditions (e.g., altering pH, temperature, co-factor) led to the conclusion that this enzyme did not possess glucuronate to glucarate converting activity.

We subsequently turned our attention towards the identification of uronate dehydrogenase from *P. syringae* using a growth-based screen. We previously observed a growth difference in *E. coli* strain DH10B on glucaric and glucuronic acid which we hoped to exploit as the basis for a genomic DNA screen to isolate the gene. As an alternative, we reviewed the metabolic pathways for growth of *E. coli* on both glucuronic and glucaric acids and determined that catabolism proceeded through two unrelated pathways. Thus, by intentionally disrupting the first step for glucuronic acid consumption (*uxa*C) while retaining the route for glucaric acid consumption, we could screen for uronate dehydrogenase activity by growth on minimal medium containing only glucuronic acid as a carbon source. Resulting cells might then contain uronate dehydrogenase, which would produce and consume glucaric acid for growth (Figure 1). Using a *uxa*C mutant harboring a *P. syringae* genomic DNA library, we successfully identified open reading frame PSPTO_1053 as coding for uronate dehydrogenase activity (deposited as GenBank Accession Number EU377538). From this sequence, homologues were identified and tested in *P. putida* and *Agrobacterium tumefaciens* (Figure 2). ***Cloning of uronate dehydrogenases was a critical step leading to assembly of the full benchmark pathway***.

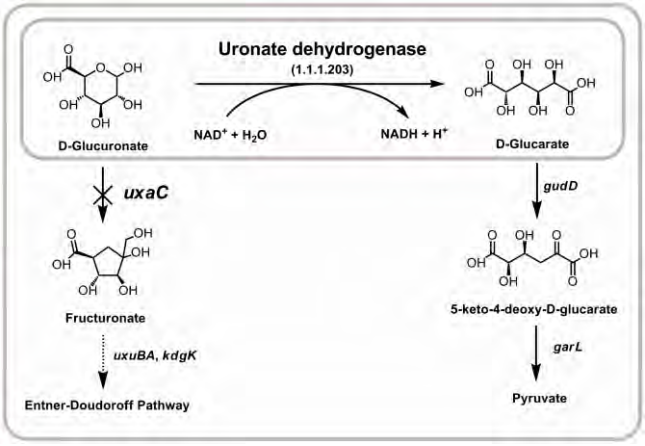

Figure 1. Catabolism of glucuronic and glucaric acids in *E. coli*

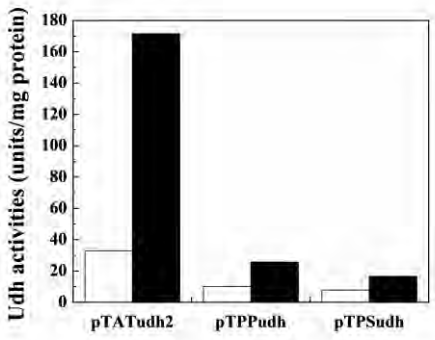

Figure 2. Uronate dehydrogenase activities of clones harboring *udh* gene of *A. tumefaciens* str. C58 (pTATudh2), *P. putida* KT2440 (pTPPudh), and *P. syringae* pv. tomato str. DC3000 (pTPSudh). Open and solid bars represent cultures grown without or with induction by 0.1 mM IPTG, respectively.

– **Assembly of the benchmark pathway** – The benchmark pathway consists of myo-inositol-1-phosphate synthase, MIPS, from yeast (*Saccharomyces cerevisiae*); myo-inositol oxygenase, MIOX, from mouse; and uronate dehydrogenase, Udh, from the bacterium *Pseudomonas syringae*, to produce glucaric acid from glucose. We had previously cloned the INO1 gene encoding MIPS through PCR amplification from the yeast genome and synthesized MIOX with codon usage for expression in *E. coli.* Both enzymes had been shown to be functionally expressed. Expression studies revealed that both enzymes needed high gene dosage levels to result in accumulation of significant amounts of their respective products (myo-inositol and glucuronic acid in the culture medium). We subsequently co-expressed the two genes, each under the control of a separate T7 promoter, and achieved production of glucuronic acid from glucose at ~0.3 g/L (Figure 3). The resulting profiles indicated that MIOX activity was rate-limiting, as evidenced by an accumulation of myo-inositol and low activity of the MIOX enzyme (data not shown).

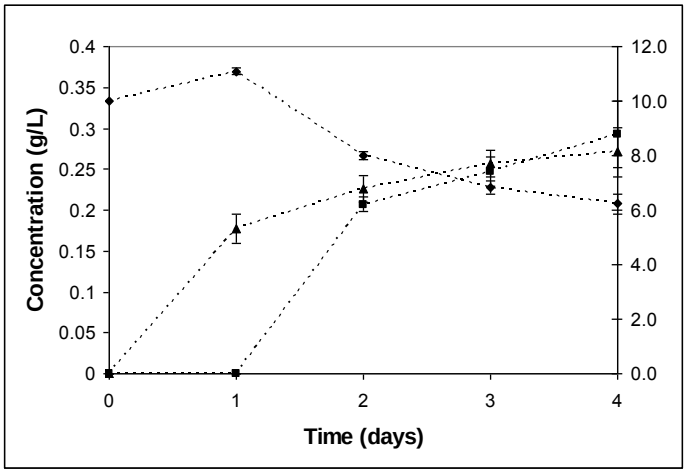

Figure 3. Production of glucuronic acid from glucose in *E. coli*. Cultures were grown in triplicate at 30°C in LB medium supplemented with 10 g/L glucose and 0.1 mM IPTG. Data points are the average and standard deviation of the three biological replicates. ▲ = Glucuronic acid; ■ = *myo*-inositol; ♦ = Glucose.

The cloned *udh* from *P. syringae* was next co-expressed with the first two enzymes of the pathway, leading to production of glucaric acid from glucose. Although the highest recombinant Udh activities were observed with the cloned gene from *A. tumefaciens*, the activity of Udh from *P. syringae* was two orders of magnitude higher than MIPS and three orders of magnitude higher than MIOX (data not shown). Thus, it was sufficient to observe glucaric acid production (Table 1). Interestingly, significantly more glucaric acid was produced in the three enzyme system than glucuronic acid produced from the two enzyme system. We believe that the high activity of Udh effectively pulls flux through the system, resulting in much higher glucaric acid concentrations. Manipulation of inducer concentrations produced a titer of ~1 g/L. ***This is the first demonstration of***

microbial production of glucaric acid, a "top value-added" product that can be produced from biomass.

Table 1. Production of glucaric acid from glucose after 3 days culture. Cultures were grown at 30°C in LB medium supplemented with 10 g/L glucose and induced with IPTG. OD_{600} = optical density at 600 nm, Yield (%) = 100 x glucaric acid produced / glucose consumed (mol/mol). Condition A = 0.1 mM IPTG at 0 hr; Condition B = 0.05 mM IPTG at 0 hr; Condition C = 0.05 mM IPTG at 0 hr and 0.1 mM IPTG at 17.5 hr. N/D = not detectable.

Condition	OD_{600}	Glucose (g/L)	*myo*-Inositol (g/L)	Glucuronic Acid (g/L)	Glucaric Acid (g/L)	Yield (%)
A	5.0	6.5	0.09	N/D	0.82	20.0
B	6.3	1.8	0.13	N/D	1.13	11.9
C	5.6	3.6	0.17	N/D	0.88	11.8

- **Improvement of productivity through co-localization of enzymes** – The accumulation of myo-inositol in the culture medium, combined with undetectable levels of glucuronic acid indicated that MIOX activity was rate-limiting in the system. Additional studies also showed that MIOX activity was strongly dependent on the presence of myo-inositol in the system. In an attempt to increase the titer, we formed a collaboration with Dr. John Dueber at the University of California, Berkeley, to utilize synthetic scaffolds to co-localize the MIPS and MIOX proteins. The MIPS and MIOX proteins were tagged with ligands, and a special scaffold was produced with the ligand-binding peptides in different stiochiometries. This results in MIPS and MIOX molecules being co-localized within the cytoplasm of the cell. Our hypothesis was that co-localization might increase the local concentration of myo-inositol (by preventing dilution by diffusion), thereby impacting the MIOX activity. Utilizing the scaffolds resulted in improvements in glucaric acid titers up to ~2 g/L. ***The use of synthetic biology parts facilitates the improvement of the system, towards titers necessary to validate biological synthesis as a suitable route for glucaric acid production.***

- **Collaboration for protein design** – In addition to the benchmark pathway utilizing naturally-occurring enzymes, we have proposed a designed pathway based only on generalized enzyme transformations. This alternative pathway requires two engineered enzymes, and we have established collaborations with Codon Devices (Cambridge, MA) and Prof. Alfonso Jaramillo at École Polytechnique (FRANCE) to obtain engineered enzymes. We received the first library, a control library, to screen for a new glucose oxidase activity from Codon Devices towards the end of the reporting period. This library of ~10,000 clones, along with a more precisely constructed library of roughly the same size will be screened in short order.

- **Modeling designed pathways** – As an off-shoot of the protein re-design project with École Polytechnique, we have also collaborated on the development of a mathematical model to predict the metabolic burden imposed by the expression of heterolgous pathways. The model is designed to both estimate the demand required to transcribe and translate plasmid-encoded genes, and to consider the impact of a heterolgous pathway on growth rate through the use of a stoichiometric, flux-balance model. This work has been described in a manuscript currently in submission. ***These types of models will be useful as a metric to choose from among many different options that will necessarily result from designed biosynthetic pathways.***

e. Work Plan – next 12 months (<500 words)

During the next 12 months, we intend to achieve the following objectives:

- **Characterize the nature of the effect of the synthetic scaffolds**. The use of synthetic scaffolds has resulted in improved production of glucaric acid. We have hypothesized that this improvement results from an increase in MIOX activity as the result of increasing the local concentration of myo-inositol, produced by the MIPS enzyme. We will further characterize these systems to understand the nature of the improvement and determine whether our hypothesis is correct. We will also evaluate a variety of scaffolds that are designed to recruit the two enzymes in different ratios.

- **Evaluation of re-designed enzymes for the designed pathway**. We received a control library from the Codon Devices during the current reporting period, and in the months since the end of that period, have also received a designed library. Both of these libraries will be screened to look for enzyme variants with higher glucose oxidase activity than that previously reported by Prof. Frances Arnold. Any hits will be characterized with respect to mutations and, with Codon Devices, will be entered into software to investigate structure-activity relationships. The successful isolation of a good glucose oxidase enzyme will facilitate the screening of an enzyme for the second reaction step. These variants will be designed by the Jaramillo group. We anticipate finalizing a delivery timelime and proceeding with construction of a library of mutants as designed by the Jaramillo group within the next reporting period.

- **Development of glucose-diverting methods**. We previously developed strains designed to allow us to establish proof-of-concept for a "glucose valve," to divert glucose between endogenous pathways and heterologous ones. We have analyzed those strains and observed some growth profiles consistent with our expectations, but have also observed some unexplained trends. Over the next 12 months, we will continue to analyze and modify these strains, until we have established an effective system for building metabolite valves.

f. Significant Problems (<250 words)

We are significantly behind our anticipated schedule for evaluating the re-engineered enzymes to be provided by our collaborators. These enzymes are necessary to functionally assemble the designed pathway. We received the first (control) library from Codon Devices for an improved glucose oxidase at the beginning of the summer, several months later than originally planned. This was due to unanticipated technical difficulties experienced by the company as they attempted to construct the synthetic DNA libraries. Likewise, receipt of re-designed enzyme variants for an aldehyde dehydrogenase from our collaborator at Ecole Polytechnique (FRANCE) has been delayed as the result of personnel issues that have delayed the computational work.

g. Technology Transfer (<500 words)

- **Leveraging of ONR funding** – The fundamental principles of retro-biosynthesis first described in the ONR proposal have subsequently been integrated into a successfully funded proposal for an NSF-sponsored Engineering Research in Synthetic Biology (SynBERC). Since the end of ONR support on 31May2008, this project has been supported by SynBERC. The MIT-France seed grant described in the following section was also obtained as the direct result of the ONR-funded work.

- **Future plans for technology transfer** – Glucaric acid has been identified as a "top-valued added" product from biomass. We anticipate that continued improvements in production of this compound through biological means will generate interest from biomass companies.

h. Foreign Collaborations and Supported Foreign Nationals

- **Collaborations** – We have established a collaboration with Prof. Alfonso Jaramillo of École Polytechnique (FRANCE) for the computational design of an aldehyde dehydrogenase capable of converting glucodialdose to glucuronic acid. This collaboration was initially established through a small seed grant provided by the MIT-France Program ($10,000, 1-year). Funds were provided to facilitate travel between France and the US. A secondary project from this collaboration resulted in the development of a mathematical model to estimate the metabolic burden imposed by the expression of designed, heterologous pathways in *E. coli*.

- **Supported Foreign Nationals** – Tae Seok Moon, a national of South Korea, is a graduate student supported by these funds during the reporting period.